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(54) Title: USE OF BETA2GPI IN DIAGNOSTIC TESTS FOR AUTOIMMUNE DISEASES

(57) Abstract: Methods and kits for *in vivo* and *in vitro* diagnosis of autoimmune diseases, including Antiphospholipid Syndrome, using β-2-glycoprotein I (β2GPI) or a fragment or variant thereof as a stimulant for cell mediated immunity. Cell mediated immune response to β2GPI is used in the diagnosis of the autoimmune disease. Methods for reducing or inhibiting cell mediated immune response to β2GPI by using fragments or analogues of β2GPI which compete for binding ligands with β2GPI.

"Use of β2GPI in diagnostic tests for autoimmune diseases"

FIELD OF THE INVENTION

The present invention relates to the use of β2GPI in the diagnosis of autoimmune diseases, and in particular it relates to an *in vitro* assay method and kit for such diagnosis.

BACKGROUND ART

The Antiphospholipid Syndrome (APS) is the most common acquired cause of predisposition to recurrent thromboembolism and management often requires lifelong high dose anticoagulation [4]. The APS is characterised by thromboembolic events, thrombocytopenia, or recurrent foetal loss in association with antiphospholipid (aPL) antibodies, immunoglobulins now recognised to be reactive with the phospholipid-binding protein, β2glycoprotein I (β2GPI) [1,2]. Although there is strong epidemiological evidence for an association between aPL antibodies and the APS [1], two issues remain problematic. The first relates to the difficulty clinicians face in defining a risk of the APS in an individual with aPL antibodies, particularly if there is no history of a thrombotic event or pregnancy failure, since their presence provides a relatively low predictive value for determining the risk of future events [5]. Population studies provide some evidence that the IgG isotype and level of aPL antibodies correlate with an increased risk for clinical complications of the APS [6,7], but this is controversial. Detection of antibodies with defined specificity for β2GPI may be more specific [8-10], but anti-β2GPI assays lack standardisation, and are not currently used in routine patient care.

The second problem is a lack of understanding about the mechanisms that cause a prothrombotic diathesis. The traditional paradigm is that aPL antibodies themselves mediate thrombosis, but despite investigations of various aspects of both procoagulant and anticoagulant pathways, no consistent abnormalities have been found [11]. Although β2GPI is clearly a strong auto-antigen that stimulates a vigorous B cell-humoral response, little attention has been focused on cellular immunity to this antigen.

SUMMARY OF THE INVENTION

The present inventors have now found that patients with autoimmune diseases have cellular immunity to β2GPI. In addition, the present inventors have found an absolute correlation between APS and β2GPI-induced monocyte procoagulant activity (PCA) in patients with aPL antibodies. These results indicate that positive cellular immunity to β2GPI, as measured by monocyte PCA, is a useful indicator of risk of thrombosis or foetal loss in patients who have aPL antibodies. β2GPI has been found by the present inventors to act as an antigen to stimulate T cell immunity and products therefrom have been found to induce procoagulants, providing a mechanism for thrombotic events in these patients.

Accordingly, in a first aspect, the present invention provides an *in vitro* method for diagnosing an autoimmune disease in a subject which method comprises:

incubating a sample of peripheral blood mononuclear cells (PBMCs) from the subject with β2GPI; and

detecting a cell mediated immune (CMI) response to the β2GPI.

The CMI response may be detected by any suitable means. For example, the CMI response may be detected by measurement of factors which cause changes in procoagulant cellular antigens produced as a result of the CMI response to β2GPI. Cellular antigens which may be monitored include tissue factor induction on monocytes.

In a second aspect, the present invention provides an *in vivo* method for diagnosing an autoimmune disease in a subject which method comprises:

injecting a sample of β2GPI intradermally to the subject; and

detecting a delayed-type hypersensitivity response to the β2GPI at the site of injection.

In a third aspect, the present invention provides a method for diagnosing APS in a subject which method comprises

incubating a sample of peripheral blood mononuclear cells (PBMCs) from the subject with β2GPI; and

detecting the monocyte procoagulant activity (PCA) of the PBMCs.

It will be appreciated that in the context of the method of the third aspect, an increase in PCA in the sample, when compared to a β2GPI-stimulated PBMC sample derived from a subject without APS, is indicative of APS.

In a fourth aspect, the present invention provides a kit for diagnosing an autoimmune disease in a subject which kit comprises

β2GPI; and

a means for detecting a cell mediated immune response.

In a fifth aspect, the present invention provides a kit for diagnosing APS in a subject which kit comprises

β2GPI; and

a means for detecting monocyte procoagulant activity (PCA).

In a sixth aspect the present invention provides a method for reducing or inhibiting the CMI response to β2GPI in a subject which method comprises administering to the subject an analogue of β2GPI, wherein the analogue competes with native β2GPI for binding ligands but which effects a reduced CMI response *in vivo* when compared to the native β2GPI.

In a seventh aspect the present invention provides a method of detecting monocyte procoagulant activity (PCA) which method comprises

contacting peripheral blood mononuclear cells (PBMCs) with β2GPI; and

detecting the monocyte procoagulant activity (PCA), **or procoagulant antigen,** of the PBMCs.

In an eighth aspect the present invention provides a method of detecting monocyte procoagulant activity (PCA) **or procoagulant antigen,** which method comprises:

(i) contacting a blood sample with an amount of water and for a period of time sufficient to lyse red blood cells

(ii) contacting white blood cells (WBC) obtained in step (i) with β2GPI; and

(iii) detecting the monocyte procoagulant activity (PCA) or procoagulant antigen, of the WBC.

In the context of the present invention, the sample of PBMCs or WBC need not be a purified sample. For example, the sample may comprise other blood products, or may consist of a sample of whole blood obtained from the subject. In a preferred embodiment, however, the sample of PBMCs is isolated from whole blood and is serum-free.

When used herein the term "β2GPI" is intended to encompass β2GPI in substantially purified native, recombinant or synthetic form. The term is also intended to encompass mutants, variants and fragments of β2GPI which retain, at least partially, an antigenic or a functional characteristic of native β2GPI.

The detection of PCA may be performed by any suitable means, for example, by plasma recalcification time as described herein, or other suitable fibrin-clot based assays. Alternatively, the PCA may be detected by an enzyme-based assay to determine the level of a product derived from the activation of coagulation or from activation of defibrogenated plasma.

In a preferred embodiment, the PCA is detected by measuring the induction of expression of monocyte tissue factor (TF).

TF expression on monocytes may be measured by any suitable means. For example, TF expression may be measured by immunodetection (for example, ELISA, flow cytometry, Western or dot blotting, or immunohistochemistry) using antibodies directed against TF and/or Factor VII bound to TF. In other embodiment, tagged factor VII/VIIa (or mutants, peptides or analogues derived therefrom) may be used to detect TF levels by ligand binding. In yet another embodiment, TF induction may be measured by polymerase chain reaction (PCR) or other molecular biological assays to detect gene expression (eg. mRNA levels) in monocytes in response to β2GPI.

The induction of monocyte TF after stimulation with β2GPI has been shown to correlate with the APS in patients with aPL antibodies. The development of the *in vitro* assay of the present invention will therefore provide a simple diagnostic test for use in the field.

In view of the findings by the present inventors, it is envisaged that β2GPI may also be used in the *ex vivo* treatment of autoimmune diseases. For example, β2GPI may

be used to select and deplete memory T cells involved in CMI from blood, bone marrow, or other tissue samples derived from patients. The depleted tissue samples may then be returned to the patient. Such *ex vivo* methods of treatment are also provided by the present invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Monocyte PCA levels in response to stimulation with β2GPI (a) or LPS (b) in PBMCs from TF-responders (TF-R), TF-non-responders (TF-NR), normal (N) and autoimmune (A) controls. Significant differences were found between TF-R and TF-NR, or controls in response to β2GPI ($p<0.0001$). Data points represent the mean of triplicate measurements. Horizontal bars represent mean values for each group. There were no significant differences between TF-R, TF-NR, A or N in response to stimulation with LPS.

Figure 2. Dose response of patient monocyte PCA induction by β2GPI. PBMCs from 3 high (P3,P4,P7) and 2 low (P9,P12) TF-responders were cultured with different amounts of β2GPI, after which monocyte PCA was measured. Data represent the mean of triplicate measurements.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Materials and Methods

Patients and blood samples

Thirty-three patients (31 female and 2 male) with anti-phospholipid antibodies (aPL) antibodies were studied. Thirty-one had aCL antibodies of either IgG or IgM isotype (measured using a standardised kit from Medical Innovations, Sydney, Australia), 2 had LA without aCL antibodies, and 7 had both LA and aCL antibodies. Twenty of the 33 had the APS with a history of thromboses and/or recurrent miscarriages whereas 13 had aPL antibodies but no history of clinical events. Seven patients with various autoimmune diseases (systemic lupus erythematosus (SLE),

psoriatic arthritis, rheumatoid arthritis or giant cell arthritis) and 7 healthy controls (both groups aCL antibody negative) were also studied. The median ages for the three groups were 43, 53 and 33 respectively. Blood was collected in citrate dextrose, diluted 1:3 with PBS, layered over Lymphoprep (Nycomed Pharma, Oslo, Norway) and centrifuged at 1800 rpm. PBMCs were collected, washed twice in PBS and resuspended at 1 x 10^7/ml in RPMI 1640 (Gibco BRL,Gaithersburg, MD) plus 20% foetal calf serum (FCS) (Gibco BRL,Gaithersburg, MD), then frozen in 7.5% dimethyl sulfoxide and stored in liquid nitrogen until further use.

Cells and incubations

PBMCs from patients, normal (N) and autoimmune (A) controls were isolated and resuspended at 1.5 X 10^6/ml in AIM V serum-free medium (Gibco BRL). β2GPI was purified by standard methods [3] under endotoxin-minimising conditions and filtered sequentially through four Zetapore syringe filters (Cuno, Meriden, CT). Endotoxin levels were measured using the Limulus amebocyte lysate assay (Pyrotell, Woods Hole, MA) to ensure that levels were < 0.125 endotoxin units. PBMCs (1.5 X 10^6 in 100 ml AIMV) were cultured with 100 µl β2GPI (25 µg/ml), or LPS (1 µg/ml) (Sigma, St Louis, MO) in 96 well round bottom plates (NUNC) and incubated at 37°C in 5% CO_2 in air for 24 h. Plates were centrifuged at 1400 rpm, supernatants removed, PBMCs resuspended in fresh AIMV (200 µl/well) and plates stored at –20°C. In some experiments, PBMCs from high (n=3) and low (n=2) responders were cultured with different concentrations of β2GPI ranging from 1 to 25 µg/ml. To determine if monocyte PCA was lymphocyte dependent, PBMCs from responders (n=2) were incubated at 2 X 10^6/ml in a flat bottom 24 well plate in RPMI plus 10% FCS for 2 h at 37°C. Nonadherent cells were removed, and adherent monocytes removed with the addition of warm (37°C) AIM V medium. Monocyte and lymphocyte populations were centrifuged, resuspended in a small volume of AIM V and counted. For induction of PCA, lymphocytes (1 X 10^6/ml) or monocytes (2 X 10^5/ml), or indicated combinations were incubated in the presence or absence of β2GPI (25 µg/ml) at 37°C in 5% CO_2 in air for 24 h, before assessment of PCA. In some experiments, PBMCs were treated with 84 µg/ml anti-HLA class II antibody (anti-DP, DQ, DR) (Serotec, Raleigh, CA), or an

irrelevant mouse IgG antibody (DAKO, Denmark) during culture with β2GPI as previously described [3].

Alternative Method for Preparing White Blood Cells

1. Add 30ml sterile endotoxin-free water (Baxter) to a sterile 50ml Falcon tube.
2. Add 1ml peripheral blood collected into citrate as per above described method
3. Mix tube by inversion by hand or mechanical means for 25 seconds at room temperature
4. Add 3ml 11x concentrated sterile Hank's balanced salt solution (HBSS; $Ca^{2+}Mg^{2+}$ free) at the end of 40 seconds
5. Centrifuge at 1300 rpm for 10 minutes at 4C
6. Remove supernatant and wash cell pellet with $Ca^{2+/}Mg^{2+}$ **HBSS**
7. Add 1ml Aim V medium and count total white cells (WBC)
8. Dispense $5x10^5$ WBC into wells of a 96 well Falcon microtitre plate in a total of 0.1ml Aim V medium.
9. Add the following:

   desired amount of antigen (in 0.1ml Aim V medium) to triplicate wells

   bacterial endotoxin (LPS, type *E.coli*), final concentration of 0.1ug/ml in 0.1ml Aim V medium, to serve as the positive control
10. Incubate for 16-20 hours at 37C in an atmosphere of CO_2 in air
11. Centrifuge the plate at 1300rpm for 10 minutes and remove supernatants
12. Add 0.2ml Aim V medium and freeze plate at -20C. Thaw quickly at 37C and repeat freeze/thaw once.
13. Measure recalcification time using a mechanical fibrometer as described below.

Measurement of Monocyte Procoagulant Activity (PCA)

Total PCA of cells subjected to two cycles of freeze-thawing was determined by a one-stage plasma recalcification time with triplicate 100 µl samples added to 100 µl pre-warmed (0.03M) $CaCl_2$ and 100 µl cold citrated human platelet-poor plasma using an automatic coagulometer (Schnitger and Gross, Amelung, Germany). and the coagulation time measured. Activity was calculated from a standard curve (log-log plot) using dilutions of human brain extract as the standard; one thousand arbitrary

units corresponded to a recalcification time of 226 secs. The results were expressed as either TF units or as a ratio of TF units of stimulated cells/TF units of unstimulated cells [TF-stimulation index (TF-SI)].

Assay for TF-dependent Factor Xa generation

TF activity on lysed and intact monocytic cells was determined using a continuous fluorogenic assay [18]. One hundred µl of PBMCs from responders (n=2) were cultured with 100 µl β2GPI (25 µg/ml) or LPS (1 µg/ml) as described. Plates were centrifuged at 1300 rpm for 10 minutes and supernatants discarded. PBMCs were washed with 200 µl Hanks balanced salt solution, centrifuged as above and supernatants discarded. The final reaction concentration of reagents was 2 ng/well Factor VII, 1µg/well Factor X, and 1.6 µM Factor X fluorogenic substrate (American Diagnostica, Greenwich, CT). The plate was read at 1 minute intervals over a period of 1 h at 360 nm (reference 460 nm) using a Cytofluor Series 4000 Multiwell plate reader (Perseptive Biosystems, Framingham, MA). In some experiments, TF expression was neutralised by the addition of 4µg anti-TF antibody (American Diagnostica) or an isotype control, to stimulated PBMCs during factor Xa generation.

That tissue factor is indeed involved in the observed results an anti-TF antibody was used to confirm its role. Results are set out in Table 1 below.

Table 1: Generation of Factor Xa (ng/well) by PBMCs cultured in media alone, 25ug/ml β2GP1, or 1ug/ml LPS

Stimulus	Media	β2GP1	LPS
PBMCs	4	12	29
PBMCs + anti-TF Ab	0	0	0
PBMCs + control Ab	4	11	23

Data represent the mean factor Xa production by PBMCs from two tissue factor responder (TF-R) patients

It will be understood however that tissue factor (TF) may be measured by a number of other techniques which would be familiar to those skillled in the art. For example:

Measurement of activity:

- Generation of factor Xa, measured after addition of FVII and FX, or from defibrogenated plasma, using chromogenic or fluorogenic substrates for FXa
- Generation of thrombin from mixtures of FVII, FX and prothrombin, or from defibrogenated plasma, using chromogenic or fluorogenic substrates for thrombin
- Measurement of activated VIIa after addition of FVII

Measurement of TF antigen:

- ELISA or other immunoassay of stimulated mononuclear cells or cell lysates using antibodies to TF or to FVIIa after addition of FVII
- Flow cytometry of fixed, stimulated mononuclear cells, using antibodies to TF or to FVIIa after addition of FVII
- Radioimmunoassay of stimulated mononuclear cells or cell lysates using antibodies to TF or to FVIIa after addition of FVII
- Any assay employing labelled or "tagged" FVII (eg biotin-labelled, radio-labelled) as a read-out of TF. These would bind TF, the receptor for FVII, and therefore provide a measure of TF.
- Measurement of TF mRNA, particularly by PCR, real-time PCR methods

Other methods for measuring (directly or indirectly) tissue factor presence, content or activity

Measurement of TF mRNA by Northern, RT-PCR can be accomplished by the method described by A. Arai, H. Hirano, Y. Ueta, et al. 2000. "Detection of mononuclear cells as the source of the increased tissue factor mRNA in the liver from lipopolysaccharide-treated rats". Thrombosis Research. 97. 153-162.

Measurement of TF by generation of FXa, prothrombin to thrombin AND by generation of FIXa is described in K. A. Bauer. 1997. "Activation of the factor VII-tissue factor pathway." Thrombosis & Haemostasis. 78. 108-111.

Measurement of TF-VIIa- generated serine proteases (ie Xa, Ixa, thrombin) can be performed by fluorogenic substrates as described for example in S. Butenas, M. E. DiLorenzo K. G. Mann. 1997. Ultrasensitive fluorogenic substrates for serine proteases. Thrombosis & Haemostasis. 78. 1193-1201.

A method using amplification of the TF-VIIa response by addition of FV/FVa can also be used and is described by S. Butenas, C. van 't Veer K. G. Mann. 1997. "Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases." Journal of Biological Chemistry. 272. 21527-21533.

Phospholipid mixtures may also be used to enhance the TF activity readout as phospholipid is an essential co-factor for activity. This approach is described in D. D. Callas, P. Bacher J. Fareed. 1995. "Studies on the thrombogenic effects of recombinant tissue factor. In vivo versus ex vivo findings." Seminars in Thrombosis & Hemostasis. 21. 166-176.

Measurements can also be made of a cellular activation signal (eg calcium flux which can be measured by flow cytometry) after FVII binding to cells expressing TF. For example, this approach is described in E. Camerer, J. A. Rottingen, E. Gjernes, et al. 1999. "Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the egr-1 gene". Journal of Biological Chemistry. 274. 32225-32233.

There is also the approach of using structural analogues of FVII, or binding peptides derived therefrom, that are "tagged" (eg with biotin, I^{125}) to quantitate TF receptor density on cells. This is described in J. Y. Chang, D. W. Stafford D. L. Straight. 1995. "The roles of factor VII's structural domains in tissue factor binding". Biochemistry. 34. 12227-12232.

A further method based on cell signalling by TF-FVII interaction is described by M. A. Cunningham, P. Romas, P. Hutchinson, et al. 1999. "Tissue factor and factor VIIa receptor/ligand interactions induce proinflammatory effects in macrophages." Blood. 94. 3413-3420.

Immunological methods to detect TF antigen:ELISA, quantitation of cells on slides (immunohistochemistry) and flow cytometry are described, for example, by B. Jude, S. Susen, B. Flan, et al. 1995. "Detection of monocyte tissue factor after endotoxin stimulation: comparison of one functional and three immunological methods." Thrombosis Research. 79. 65-72.

The use of chromogenic substrates for serine proteases generated by TF-VIIa. Also, measurement of FVII antigen levels bound to cell TF and/or assessed by

ELISA/flow cytometry, as well as the potential use of factors derived from other species (eg bovine) in the assays, is described by K. Kario, T. Matsuo, R. Asada, et al. 1995. "The strong positive correlation between factor VII clotting activity using bovine thromboplastin and the activated factor VII level". Thrombosis & Haemostasis. 73. 429-434.

TF measurement can also be performed in urine. Measurements of TF can also be made in blood plasma (ie not on cells). This may be relevant to measure in patients undergoing thrombotic episodes in the populations. These approaches are described in B. A. Lwaleed, J. L. Francis M. Chisholm. 1999. "Tissue factor assays as diagnostic tools for cancer? Correlation between urinary and monocyte tissue factor activity." Journal of Hematotherapy & Stem Cell Research. 8. 659-668.

TF antigen assay can be performed by Western blotting and flow cytometry. Detection of TF by immunodetection may be by monoclonal or polyclonal antibodies to TF, or to FVII which is bound to TF on cells. Such an approach is described in S. R. Meisel, I. Shimon, T. S. Edgington, et al. 1999. "Leukaemia inhibitory factor enhances tissue factor expression in human monocyte-derived macrophages: a gp130-mediated mechanism." British Journal of Haematology. 107. 747-755.

A novel technique of using FV as a readout of TF-VIIa activity may also be employed. This technique also indicates use of phospholipids/vesicles as TF cofactor and is described by O. Safa, J. H. Morrissey, C. T. Esmon, et al. 1999. "Factor VIIa/tissue factor generates a form of factor V with unchanged specific activity, resistance to activation by thrombin, and increased sensitivity to activated protein C." Biochemistry. 38. 1829-1837.

Detection of fibrin formation in whole blood may also be used, as described in R. A. Santucci, J. Erlich, J. Labriola, et al. 2000. Measurement of tissue factor activity in whole blood." Thrombosis & Haemostasis. 83. 445-454.

Use can also be made of an active-site inhibited FVIIa that binds TF with higher affinity than FVII and could be used as a "tag", if labelled, to quantitate TF available on cells (B. B. Sorensen L. V. Rao. 1998. Interaction of activated factor VII and active

site-inhibited activated factor VII with tissue factor." Blood Coagulation & Fibrinolysis. 9. S67-71.)

Other useful procedures for measuring TF are described in, for example, Luther T., Flossel, C., Hietschold V et al., 1990: "Flow cytometric analysis of tissue factor (TF) expression on stimulated monocytes-comparison of procoagulant activity of mononuclear cells". Blut: 61, 375-8; Amirkhosravi A., Alexander M., May K. et al., 1996: "The importance of platelets in the expression of monocyte TF antigen measured by a new whole blood flow cytometric assay." Thrombosis and Haemostasis 75: 87-95; Iochmann S., Reverdiau-Moalic P., Beaujean S., et al., 1999: "Fast detection of tissue factor and tissue factor pathway inhibitor messenger RNA in endothelial cells and monocytes by a sensitive reverse transcription polymerase chain reaction." Thrombosis Research: 94: 1657-73; Morrissey J., Fair D., Edgington T., 1988: "Monoclonal antibody analysis of purified and cell-associated tissue factor. Thrombosis Research: 52: 247-61

Certain embodiments of the present invention contemplate using reconstitution of cellular components necessary to generate the appropriate signal. Thus in a particular embodiment white blood cell or peripheral blood polymorphonyclear cell preparations may be supplemented with platelets and/or neutrophils (granulocytes) in the assays (Halvorsen H., Olsen J., Osterud B ., et al., 1993: "Granulocytes enhance LPS-induced tissue factor activity in monocytes via an interaction with platelets" J. Leukocyte Biology 54: 275-82.)

Methods utilising measurements of thrombin generation in whole defibrinogenated plasma, by using a thrombin-specific chromogenic substrate, are also contemplated. In such methods it is very simple to remove the fibrinogen from plasma and make the assay much more economical than adding pure coagulation factors like FVII and FX (Lando P., Biazak C., Edgington T., 1986: "Amidolytic assay for procoagulant activity of lymphoid and tumor cells." J. Immunological Methods 89: 131-39.)

Another useful immunoassay is described by Andoh K/. Kubota T., Koboyashi S., Maekawa T., 1986: "Radioimmunoassay for human tissue factor". Thrombosis Research, 43: 275-286.

An assay that uses changes in fibrinogen (pure) to fibrin as a readout of thrombin activity may also be used (Doellgast G., Rothberger H. 1985: "Enzyme-linked coagulation assay: a clot-based, solid phase assay for thrombin". Analytical Biochemistry 147: 529-534)

A further useful procedure is described by Fareed J., Messmore H., Walenga J. et al., 1983: "Diagnostic efficacy of newer synthetic-substrate methods for assessing coagulation variables" Clinical Chemistry: 29: 225-236 , whic uses synthetic substrates for Xa, thrombin, IXa.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) or standard error of the mean (SEM). Differences between patient and control groups were determined using One Way Anova analysis.

Results and Discussion

Elevated levels of plasma and monocyte-associated TF have been reported in patients with aPL antibodies [13,14], and elevated monocyte TF correlates with histories of thrombosis [14]. Thus, we hypothesised that a procoagulant diathesis in APS patients may be due to upregulation of monocyte TF as a result of activation of autoreactive CD4+ T lymphocytes. To investigate this, we measured monocyte PCA in PBMCs from 33 patients with aPL antibodies, as well as 14 aPL antibody-negative normal (N) (n=7), or autoimmune (A) (n=7) controls. PBMCs from normal or autoimmune controls generated low levels of monocyte PCA in response to β2GPI (mean ± SEM TF-SI 5 ± 1 or 3 ± 1 respectively). In contrast, β2GPI incubated with PBMCs from patients with aPL antibodies upregulated monocyte PCA in 20 of 33 patients, as defined by a TF-SI of >4 SD above the mean response of normal controls to β2GPI (>14.14). These 20 patients were designated TF-responders (TF-R) and the remaining 13 TF-non-responders (TF-NR) (Fig. 1a). A highly significant difference in mean monocyte PCA levels generated in response to β2GPI was observed between

these two groups (80 ± 11 vs 6 ± 1, p<0.0001). Moreover, the TF-SI values for all TF-R were >25, whereas those for all TF-NR, and all controls fell below 15, distinguishing individual TF-R from all other groups. Of the 20 TF-responders, PBMCs from 8 had previously been shown to proliferate to β2GPI in vitro, whereas 10 did not [3], supporting the observation that induction of monocyte PCA is a more sensitive measure of cellular immunity than proliferation [12].

Furthermore, and most importantly, all 20 patients designated as TF-responders had histories of thrombosis or recurrent foetal loss, whereas the 13 TF-non-responders had aPL antibodies but no history of these events. Thus, for the first time, we show an absolute correlation between the APS and β2GPI -induced monocyte PCA in patients with aPL antibodies. These results raise the possibility that positive cellular immunity to β2GPI, as measured by induction of monocyte TF in vitro, could be a useful indicator of risk of thrombosis or foetal loss in patients who have aPL antibodies.

The lack of induction of monocyte PCA to β2GPI by PBMCs from APS-negative patients with aPL antibodies and both control groups was not due to any inherent lack of monocyte responsiveness, since all groups showed equal upregulation of monocyte PCA when stimulated with LPS, a well-established inducer of monocyte TF. There was no significant difference (p=0.296) in TF-SI values to LPS between TF-R (37 ± 12), TF-NR (44 ± 12), normal controls (60 ± 38), or autoimmune controls (57 ± 11) (Fig. 1b). Although the 20 patients with APS had higher mean IgG-aCL antibody levels (40 ± 9 GPL units) than those without APS (24 ± 11 GPL units), differences were not statistically different (p=0.693).

Induction of PCA by β2GPI was dose-dependent when PBMC from 3 high and 2 low TF-R were tested (Fig. 2). PCA was observed upon exposure of PBMCs to as little as 6.25 μg/ml β2GPI, with maximal PCA at 12.5-25 μg/ml. The monocyte PCA activity measured in the recalcification assay was shown to be primarily due to expression of TF. β2GPI-stimulated PBMCs from two TF-R patients generated a mean of 12 ng of Factor Xa, compared to 4 ng and 29 ng following culture with media alone or LPS respectively, but inclusion of a neutralising anti-TF antibody completely abrogated Factor Xa generation, whereas the isotype control antibody had no

significant effect (11ng, 4ng and 23 ng for β2GPI, media, and LPS-stimulation respectively).

Induction of monocyte PCA by β2GPI required stimulation of β2GPI-specific lymphocytes. As shown in Table 1, monocytes alone showed no increase in PCA when stimulated with β2GPI, but the inclusion of lymphocytes with monocytes at a ratio of 5:1, induced substantial PCA, which increased further when the ratio of lymphocytes was doubled. Moreover, β2GPI-induced monocyte PCA was abrogated by treatment of PBMCs with neutralising anti-class II antibody, but not the isotype control, confirming that PCA induction required antigen presentation to CD4+ T lymphocytes (Table 2).

Table 2: Lymphocytes and class II molecules are required for the generation of monocyte PCA in response to stimulation with β2GPI

Cell population	Stimulus	TF-SI
Lymphocytes	none	0.92 ± 0.9
Monocytes	none	1.2 ± 1.2
Lymphocytes	β2GPI	1.1 ± 0.7
Monocytes	β2GPI	1.1 ± 0.7
Lymphocytes:Monocytes (5:1)	β2GPI	15.4 ± 9.5
Lymphocytes:Monocytes (9:1)	β2GPI	21.8 ± 10.1
PBMCs - unfractionated	β2GPI	49.3 ± 4.0
- plus control antibody	β2GPI	56 ± 8.0
- plus anti-HLA antibody	β2GPI	6.6 ± 0.1

Data are expressed in TF-SI units and show mean ± SEM of triplicate measurements

The results of this study suggest that patients with the APS have chronic low grade stimulation of β2GPI-specific T lymphocytes by continuous exposure to this plasma protein, leading to a persistently high expression of monocyte TF and resulting in a prothrombotic diathesis. This hypothesis is consistent with previous reports of elevated levels of plasma TF [13] and monocyte TF [14] in patients with aPL

antibodies, particularly in those patients with histories of thrombosis [14]. The mechanism by which auto-reactive CD4+ T cells induce TF in the APS is not clear, but may involve IFN-γ and other procoagulant inducing factors [19,20]. Type 1 (Th1) but not Type 2 (Th2) T-helper lymphocytes have been reported to induce monocyte TF [15].

The production of IFN-γ by activated β2GPI-specific T lymphocytes may also be a factor in the high frequency of pregnancy failure in APS patients. Normal pregnancy has been considered a Type 2 (Th2) phenomenon immunologically, with prominent production of IL-4, IL-5, and IL-10 in fetoplacental tissues [16]. Type 1 (Th1) cytokines such as IFN-γ have detrimental effects on foetal development and the ability to sustain pregnancy [17], and production of IFN-γ and related cytokines by β2GPI-specific T lymphocytes could compromise pregnancy in patients with the APS.

Results of Studies Using Alternative Method for Preparing White Blood Cells

WBC	mU TF/10^6 WBC	Activity/10^6monocytes
Unstimulated	3.4	340
LPS 10ng/ml	36.8	3680
LPS 1ng/ml	24.2	2420
LPS 100pg/ml	8.6	860
LPS 10pg/ml	6.2	620

Regarding the above results an assumption has been made that the WBC population contains only ~1% monocytes, and all TF is on monocytes. The values can be multiplied by 100 to give approximate activity/monocyte.

The above results compare with the following which are obtained with PBMC isolated by the normal procedure

PBMC	mU TF/10^6PBMC	Activity/10^6monocytes
Unstimulated	42	420
LPS 1 ng/ml	484	4840

LPS 100pg/ml	92	920
LPS 10 pg/ml	32	320

As a guide only, PBMC normally contain ~90% lymphocytes and ~10% monocytes whereas the WBC normally contain about 90% neutrophils, ~ 9% lymphocytes and ~1% monocytes.

In summary, the results of this study have shown an absolute correlation between β2GPI-specific induction of monocyte TF and clinical events of the APS, providing for the first time evidence for a uniform mechanism to explain the prothrombotic diathesis seen in patients with the APS. In addition, measurement of this autoimmune cellular response may become a clinically powerful indicator of risk of the APS in patients with aPL antibodies, and represents a significant advance in the clinical management of individual patients with this syndrome.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:

1. Method for the *in vitro* diagnosis of an autoimmune disease in a subject which method comprises:

incubating a sample of peripheral blood mononuclear cells (PBMCs) from the subject with β2GPI; and

detecting a cell mediated immune (CMI) response to β2GPI.

2. A method according to claim 1, wherein the CMI response is detected by measurement of factor(s) which cause changes in procoagulant cellular antigens produced as a result of the CMI response to β2GPI.

3. A method according to claim 2, wherein the factor is tissue factor.

4. Method for the *in vivo* diagnosis of an autoimmune disease in a subject which method comprises:

injecting a sample of β2GPI, intradermally to the subject; and

detecting a delayed-type hypersensitivity response to the β2GPI, at the site of injection.

5. Method for diagnosing Antiphospholipid Syndrome (APS) in a subject which method comprises

incubating a sample of peripheral blood mononuclear cells (PBMCs) from the subject, with β2GPI; and

detecting the monocyte procoagulant activity (PCA) or procoagulant antigen of the PBMCs, wherein an increase in PCA or procoagulant antigen in the sample, when compared to a β2GPI-stimulated PBMC sample derived from a subject without APS, is indicative of APS.

6. Method for reducing or inhibiting the CMI response to β2GPI in a subject which method comprises administering to the subject a fragment or an analogue of β2GPI, wherein the fragment or the analogue competes with native β2GPI for binding ligands but which effects a reduced CMI response *in vivo* when compared to the native β2GPI.

7. A method according to any one of claims 1 to 6, wherein the sample of PBMCs is not a purified sample.

8. A method according to any one of claims 1 to 6, wherein the sample of PBMCs is isolated from whole blood and is serum-free.

9. Kit for diagnosing an autoimmune disease in a subject which kit comprises β2GPI; and

a means for detecting a cell mediated immune response.

10. Kit for diagnosing APS in a subject which kit comprises

β2GPI; and

a means for detecting monocyte procoagulant activity (PCA) or procoagulant antigen.

11. Method of detecting monocyte procoagulant activity (PCA) or procoagulant antigen, which method comprises

contacting peripheral blood mononuclear cells (PBMCs) with β2GPI; and

detecting the monocyte procoagulant activity (PCA) or procoagulant antigen of the PBMCs.

12. Method of detecting monocyte procoagulant activity (PCA) or procoagulant antigen, which method comprises:

(i) contacting a blood sample with an amount of water and for a period of time sufficient to lyse red blood cells

(ii) contacting white blood cells (WBC) obtained in step (i) with β2GPI; and

(iii) detecting the monocyte procoagulant activity (PCA) or procoagulant antigen of the WBC.

13. A method according to claim 11 or claim 12, wherein the detecting the monocyte procoagulant activity (PCA) or procoagulant antigen is detected by measuring the induction of expression of monocyte tissue factor (TF).

14. A method according to claim 11 or claim 12, wherein the monocyte procoagulant activity (PCA) or procoagulant antigen is detected by fibrin-clot based assays.

15. A method according to claim 14, wherein the detecting the monocyte procoagulant activity (PCA) or procoagulant antigen is detected by plasma recalcification time.

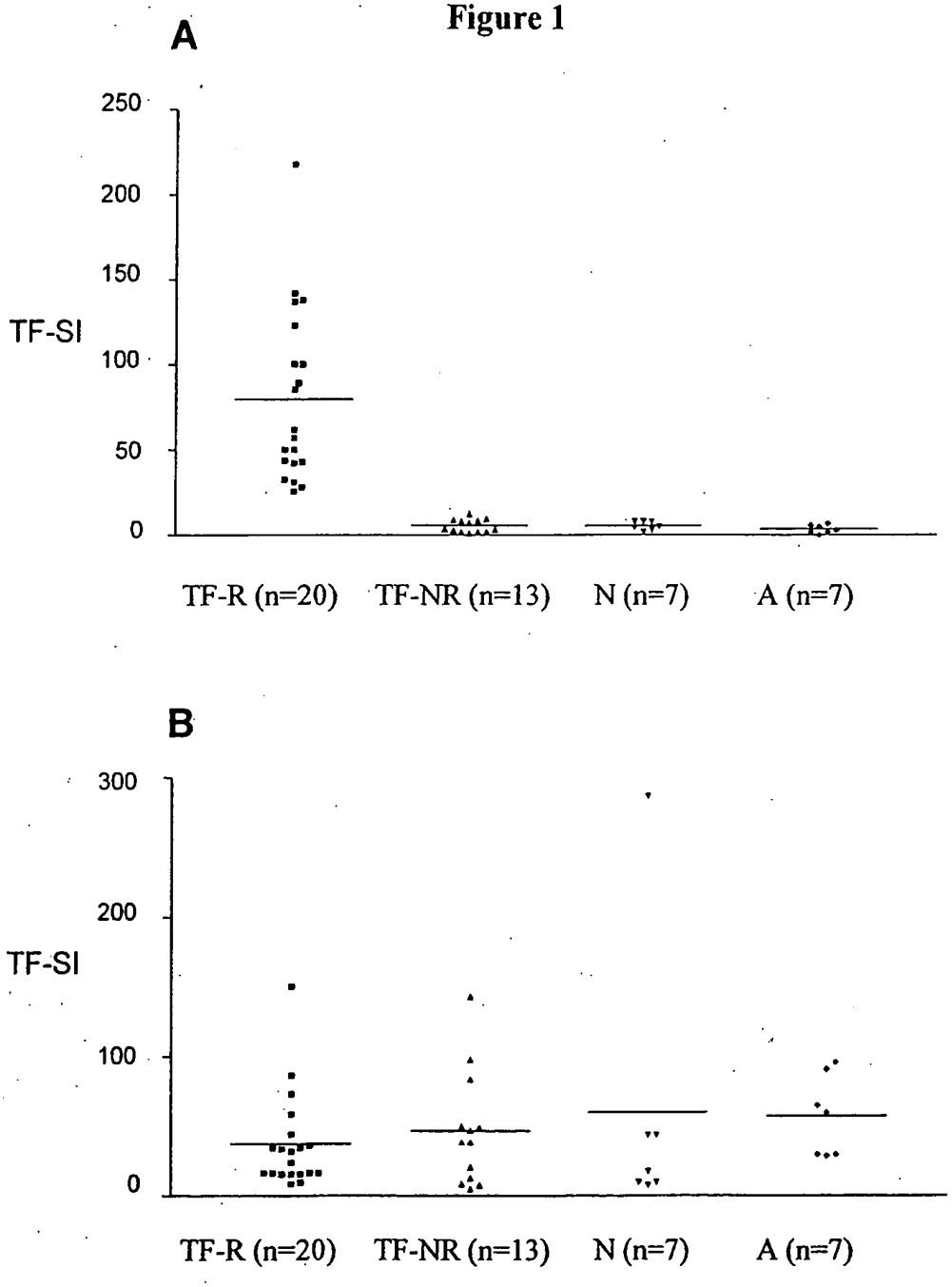
16. A method according to claim 11 or claim 12, wherein the monocyte procoagulant activity (PCA) or procoagulant antigen is detected by an enzyme-based assay to determine the level of a product derived from the activation of coagulation or from activation of defibrogenated plasma.

17. A method according to claim 13, wherein the tissue factor expression on monocytes is measured by immunodetection using antibodies directed against TF and/or Factor VII.

18. A method according to claim 13, wherein the tissue factor expression on monocytes is measured by tagged factor VII/VIIa, or mutants, peptides or analogues derived therefrom, to detect tissue factor levels by ligand binding.

19. A method according to claim 13, wherein the tissue factor expression on monocytes is measured by polymerase chain reaction (PCR) or other molecular biological assays to detect gene expression in monocytes in response to β2GPI.

Figure 1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TF-NR (n=13)
N (n=7)
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B
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100
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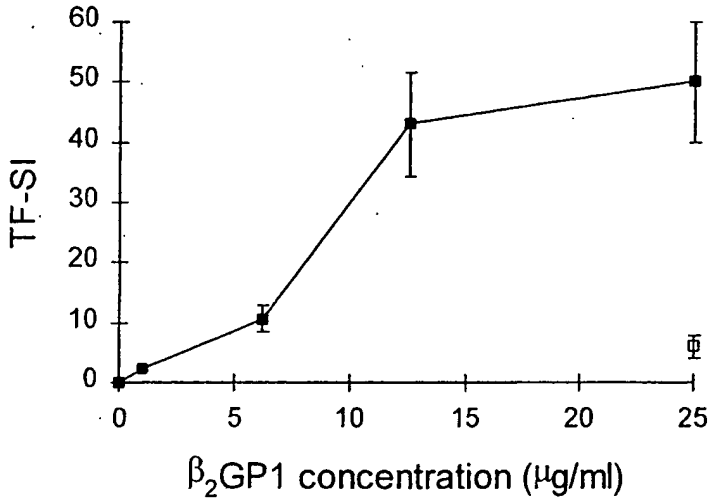
TF-SI
TF-R (n=20)
TF-NR (n=13)
N (n=7)
A (n=7)

Figure 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01068

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: G01N 33/564, A61K 39/00, A61K 49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT Chemical Abstracts (CA): Keywords (KW) see electronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Medline (ML): Keywords (KW) see electronic database box below.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, CA, ML: KW (beta 2 glycoprotein 1 or apolipoprotein H) and (antiphospholipid syndrome) and (T cell or lymphocyte or clot? or coagula? or bind? or ligand or monocyte procoagulant or tissue factor)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VISVANATHAN S *et al*, "Cellular Immunity to β_2-Glycoprotein-1 in Patients with the Antiphospholipid Syndrome," *The Journal of Immunology*, 1 June 1999, 162(11):6919-6925 whole of document	1-3, 5, 7-8, 11-19
P, X	WO 00/01729 A2 (YEDA RESEARCH AND DEVELOPMENT) 13 January 2000 whole of document	6
X	WO 96/40197 A1 (LA JOLLA PHARMACEUTICAL COMPANY) 19 December 1996 whole of document	6

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2000	31 OCT 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer GARETH COOK Telephone No : (02) 6283 2541

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01068

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLANK M *et al*, "Prevention of experimental antiphospholipid syndrome and endothelial cell activation by synthetic peptides," *Proceedings of the National Academy of Sciences*, April 1999, 96:5164-5168 whole of document	6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01068

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 9 and 10

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims are vague and unclear such that no meaningful search could be conducted. They define kits of known components which could be used in diagnosis of an autoimmune disease or APS. However no feature or arrangement is defined to limit the individual components solely to that use. Without such an arrangement or feature being defined, no definite scope can be placed on the claims to allow for a meaningful search..

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01068

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II (Lack of Unity)

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. In coming to this conclusion the International Searching Authority has found that there are different inventions as follows:

1. Claims 1 to 5, 7, 8 and 11 to 19 are directed towards methods of diagnosis of autoimmune diseases *in vivo* and *in vitro* involving exposure to β2GPI and detection of an immune response.

2. Claim 6 is directed towards a method for reducing or inhibiting an immune response to β2GPI involving the use of analogues or fragments of β2GPI.

Due to the fact that β2GPI and its analogues are known compounds, the above inventions do not share a single inventive concept. For this reason this international application does not comply with the requirements of unity of invention.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01068

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	00/01729	AU	46442/99				
WO	96/40197	AU	62710/96	CN	1 192 153	JP	11 507 822
		CA	2 223 687	CN	1 225 015	EP	833 648
		US	5 874 409				

END OF ANNEX